

A Multidrug Resistant Engineered CAR T Cell for Allogeneic Combination Immunotherapy

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Abstract

The adoptive transfer of CAR T cell represents a highly promising strategy to fight against multiple cancers. The clinical outcome of such therapies is intimately linked to the ability of effector cells to engraft, proliferate and specifically kill tumor cells within patients. When allogeneic CAR T cell infusion is considered, host versus graft and graft versus host reactions must be avoided to elicit successful antitumor activity *in vivo*. We propose to address these requirements through the development of multidrug resistant TCR $\alpha\beta$ -deficient CAR T cells (CAR T cells KO D/T). We demonstrate that CAR T cells KO D/T are fully TCR deficient, display efficient antitumor activity and proliferate in the presence of purine nucleotide analogues (PNA), currently used in clinic as preconditioning lymphodepleting regimens. We envision that CAR T cells KO D/T could be generated from third party healthy donors and used in any patients as antitumor allogeneic immunotherapy without generating TCR $\alpha\beta$ -dependent GvH reaction. Their drug resistance properties could enable them to resist to simultaneous infusion of lymphodepleting regimens to inhibit the host immune system and control their rate of ablation via HvG reaction.

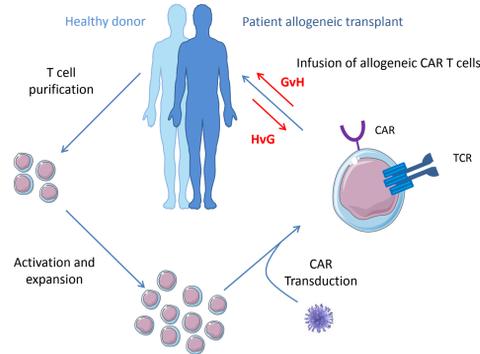


Figure 1. Principle of allogeneic CAR T cell immunotherapy illustrating graft versus host (GvH) and host versus graft (HvG) reactions that may occur after allogeneic transfer of CAR T cells

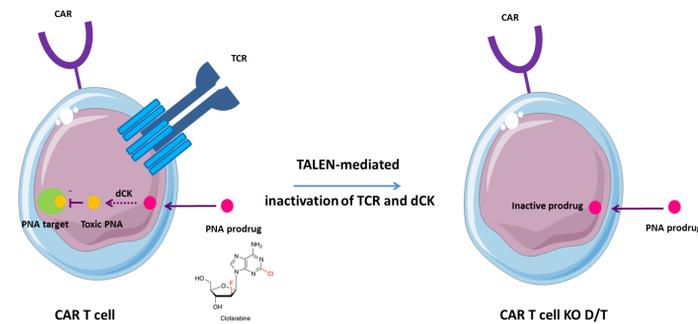


Figure 2. Genome engineering strategy to avoid HvG and GvH reactions. This strategy consists in the inactivation of TRAC and dCK (deoxycytidine kinase) genes respectively responsible for TCR $\alpha\beta$ surface expression and purine nucleotide analogues (PNA) toxicity. Efficient depletion of TCR at the surface of CAR T cells is expected to prevent them from attacking host tissues via the TCR-dependent GvH reaction. dCK deficiency could enable them to resist to simultaneous infusion of lymphodepleting dose of PNA to inhibit the host immune system and control their rate of ablation via HvG reaction.

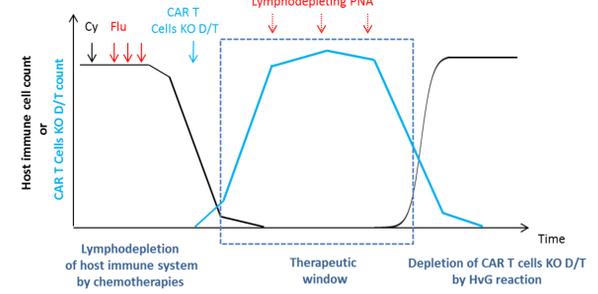


Figure 3. Schematic representation of the allogeneic adoptive transfer therapeutic window.

TALEN-mediated TRAC/dCK gene processing is efficient in primary T cells

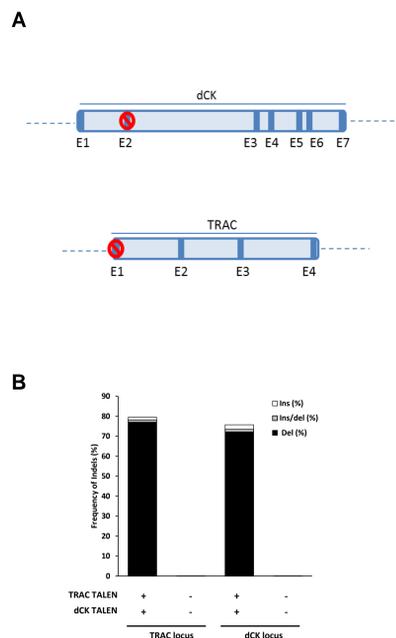


Figure 4. TALEN treatment of primary T cells enables simultaneous and highly efficient processing of TRAC and dCK genes. (a) Overall gene architectures of dCK and TRAC open reading frames. Exons (dark blue) and introns (light blue) as well as the location of TALEN-mediated gene processing (red mark) are indicated. (b) Genotypic characterization of TALEN-mediated inactivation of dCK and TRAC genes by high throughput DNA sequencing. T cells treated with dCK and TRAC TALEN were allowed to grow for 6 days, their genomic DNA was extracted, amplified using TRAC or dCK specific PCR amplicons and analysed by high throughput DNA sequencing. The frequencies of indels generated at TRAC and dCK locus are indicated.

TALEN-mediated TRAC gene processing disrupts TCR $\alpha\beta$ expression at the surface of primary T cells

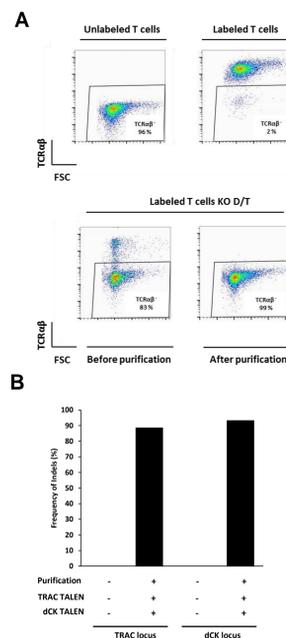


Figure 5. Multiplex TALEN treatment of primary T cells disrupts TCR membrane expression. (a) Purification of TRAC KO T cells from the bulk population of cell transfected with mRNA encoding TRAC and dCK TALEN. Labeling control experiment performed with T cells in the presence or in the absence of anti-TCR mAb (upper panel) and monitoring of TCR $\alpha\beta$ -deficient cells gathered before and after TRAC KO T cells purification using anti-CD3 magnetic beads and MACS LD-column (lower panel). (b) Genotypic characterization of TALEN-mediated inactivation of TRAC and dCK genes by high throughput DNA sequencing before and after TCR negative cells purification.

TALEN-treated CAR T cells are resistant to multiple PNAs and are able to proliferate in the presence of their clinically relevant dose

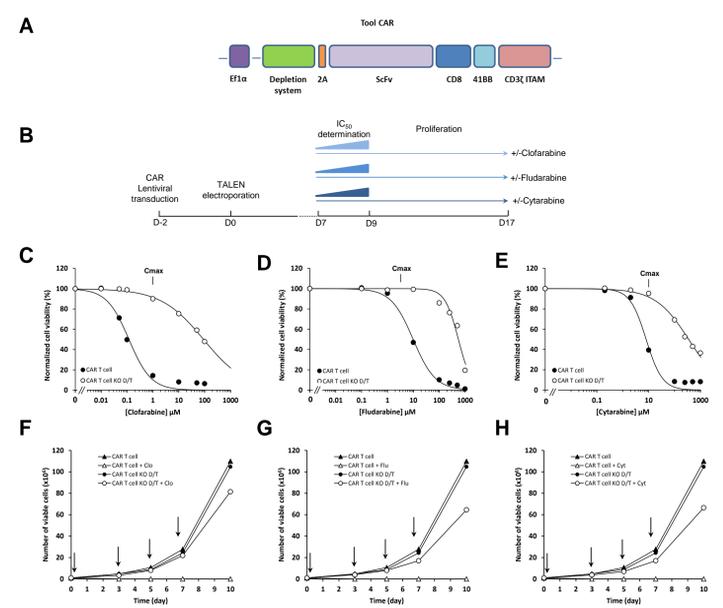


Figure 6. TALEN-treated CAR T cells are resistant to lethal dose of different purine and pyrimidine nucleotide analogues. (a) Scheme of the polycistronic DNA expression cassette encoding the membrane exposed rituximab-dependent depletion system (RQR8), followed by a 2A cis-acting hydrolyase element and a tool CAR. (b) Scheme of the experimental workflow used to engineer and characterize T cells proliferative capacity and resistance properties toward clofarabine, fludarabine and Cytarabine. (c, d and e) Clofarabine, fludarabine and cytarabine IC_{50} determination. Experimental viability values obtained after 2 days of incubation were normalized with respect to the one obtained in the absence of drug. (f, g and h). Proliferation capacity of CAR T cells in the presence or in the absence of clofarabine, fludarabine and cytarabine. 10^6 CAR T cells were grown for a total of 10 days in the absence or in the presence of 1, 5 and 10 μ M of clofarabine, fludarabine and cytarabine respectively. Vertical arrows indicate each passage step where cells were diluted to 10^6 cells/mL in the presence or in the absence of drug.

TALEN-treated CAR T cells display efficient antitumor activity

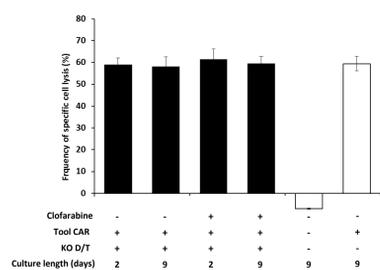


Figure 8. TALEN-treated CAR T cells retain their cytolytic activity after 2 to 9 days of culture in the presence of clinically relevant dose of clofarabine. 10^6 Daudi (target cells) and 10^5 K562 (negative control) cells were co-cultured with CellTrace™ CFSE and CellTrace™ violet were co-cultured in 100 μ l for 5h at 37°C with 10^6 of TALEN-treated CAR T cells grown for 2 or 9 days in the presence or in the absence of 1 μ M clofarabine. Cells were then recovered and their viability was determined by flow cytometry. Viability of Daudi and K562 cells were used to calculate the frequencies of specific cell lysis (lanes 1 to 4). T cells and CAR T cells cultured in the absence of clofarabine for 9 days were respectively used as negative and positive controls of specific cell lysis activity (lanes 5 and 6). Results are displayed as specific cell lysis activities relative to the control experiment performed with CAR T cells. Error bars represent the mean of 2 independent experiments + SD.

TALEN-treated CAR T cells can pair up with PNA chemotherapy for better antitumor activity

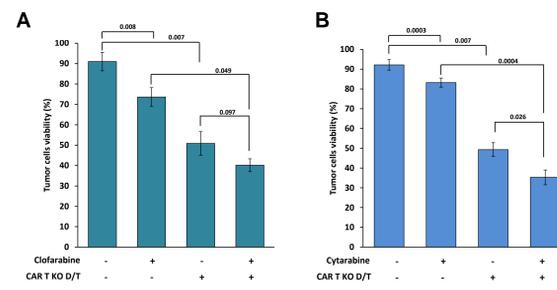


Figure 9. TALEN-treated CAR T cells can pair up with chemotherapy for better antitumor activity. 10^6 Daudi cells (target cells) preincubated in the presence or in the absence of PNA (1 μ M clofarabine or 10 μ M cytarabine) for 24h, were labeled with CellTrace™ CFSE and co-cultured in 100 μ l for 5h at 37°C in the absence or the presence of 10^6 TALEN-treated CAR T cells (CAR T KO D/T). Cells were then recovered and their viability was determined by flow cytometry. The residual viability of tumor cells (Daudi) obtained in the presence or in the absence of clofarabine (a) or cytarabine (b) are displayed. p values numbers, calculated according to the procedure described in material and methods, are indicated. Error bars represent the mean of 2 or 3 independent experiments +/- SD performed in the presence of clofarabine or cytarabine respectively.

Conclusions

In summary, we report the development of multidrug resistant and TCR $\alpha\beta$ -deficient CAR T cells compatible with allogeneic immunotherapy. Engineered CAR T cells resistance towards PNA chemotherapies enabled them to proliferate under lymphodepleting conditions while retaining their antitumor activity and specificity. Such PNA resistance provides two main advantages. First, it could enable allogeneically transferred CAR T cells to survive lymphodepleting regimens used to maintain the host immune system in check and control their rate of ablation via a HvG reaction. Second, it could allow combination therapy, an approach likely to improve clinical outcomes by potentializing CAR T cell antitumor activity *in vivo*. In addition, due to their lack of surface exposed-TCR, these cells could be theoretically generated from third party healthy donors and used as allogeneic immunotherapy without generating GvH reaction. In conclusion, we believe that the cellular engineering strategy described in this work will provide a basic frame work to generate a universal T cell that will foster large scale utilization of ACT and thus could benefit a broader range of patients.

Allogenic CAR T-cells Targeting CD123 for Adoptive Immunotherapy of Acute Myeloid Leukemia (AML)

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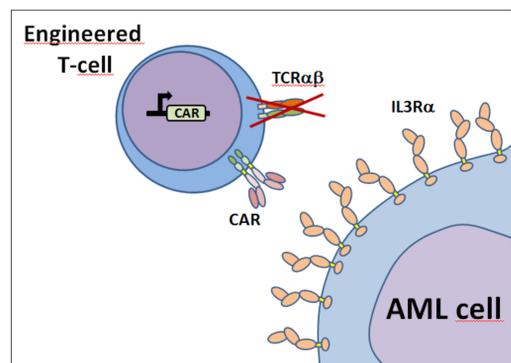
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#1 Abstract

Chimeric antigen receptor (CAR)-redirected T-cells have given rise to long-term durable remissions and remarkable objective response rates in patients with refractory leukemia, raising hopes that a wider application of CAR technology may lead to a new paradigm in cancer treatment. A limitation of the current autologous approach is that CAR T-cells must be manufactured on a "per patient basis". We have developed a standardized platform for manufacturing T-cells from third-party healthy donors to generate allogeneic "off-the-shelf" engineered CAR+ T-cell-based frozen products. This platform utilizes Transcription Activator-Like Effector Nuclease (TALEN) gene editing technology to inactivate the TCR α constant (TRAC) gene, eliminating the potential for T-cells bearing alloreactive TCR's to mediate Graft versus Host Disease (GvHD). We have previously demonstrated that editing of the TRAC gene can be achieved at high frequencies, obtaining up to 80% of TCR α negative cells. This allows us to efficiently produce TCR-deficient T-cells that have been shown to no longer mediate alloreactivity in a xeno-GvHD mouse model. In addition to CAR expression, our T-cells are engineered to co-express the RQR8 gene as a safety feature, with the aim of rendering them sensitive to the monoclonal antibody rituximab.

In this work we present the adaptation of this allogeneic platform to the production of T cells targeting CD123, the transmembrane alpha chain of the interleukin-3 receptor, which is expressed in tumor cells from the majority of patients with Acute Myeloid Leukemia (AML). To identify an effective CAR targeting CD123, we have screened multiple antigen recognition domains in the context of several different CAR architectures to identify candidates displaying activity against cell lines expressing variable levels of the CD123 antigen. Furthermore, experiments in an AML mouse model using anti-CD123 CAR T cells demonstrate important anti-tumor activity in vivo. The ability to carry out large scale manufacturing of allogeneic, non alloreactive CD123 specific T cells from a single healthy donor will thus offer the possibility of an off-the-shelf treatment that would be immediately available for administration to a large number of AML patients.

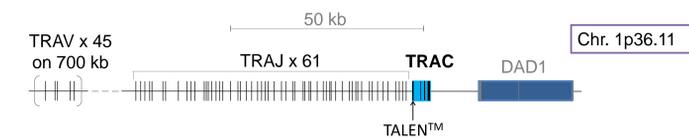
#2 Allogenic approach targeting CD123 to treat AML



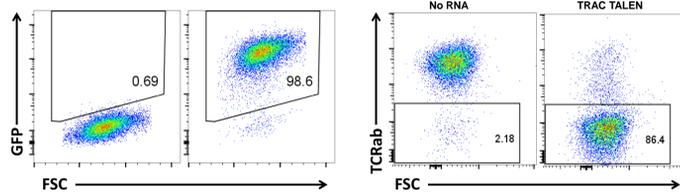
One of the key barriers to the adoptive transfer of 3rd party CAR T-cells can be overcome via the application of TALEN gene editing technology, deletion of the TCR gene and thus the use of any donors' T-cells without the risk of GvHD.

#3 TCR alpha inactivation prevents Graft vs. Host disease

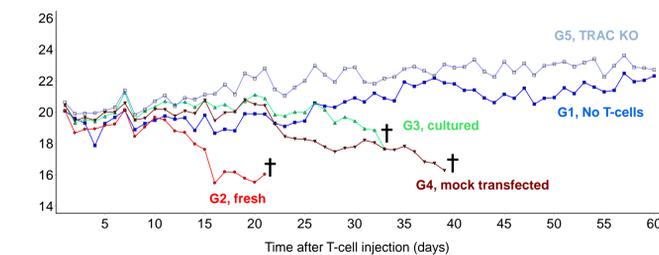
TALEN were designed to recognize and cleave the coding sequence of human TCR α gene



High levels of gene inactivation can be obtained in primary T cells by electrotransfer of TALEN mRNAs using a PulseAgile[®] electroporation technology.



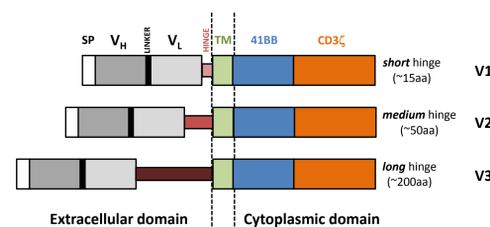
Xenogeneic model of Graft vs. Host Disease: Irradiated NOG mice were injected with PBS (group 1), or 30x10⁶ human T cells from the same donor. The cells were obtained from activated PBMCs after 3 day expansion (group 2), 15-day expansion (group 3), mock electroporation and 15-day expansion (group 4), or TRAC TALEN-electroporation, 15-day expansion and depletion of TCR+ cells (group 5).



No mice receiving TRAC TALEN-treated cells showed signs of GvHD, whereas all mice receiving non engineered cells developed GvHD and had to be sacrificed. Human cells survived at least 2 weeks in all groups.

#4 Generation of anti-CD123 Chimeric Antigen Receptors

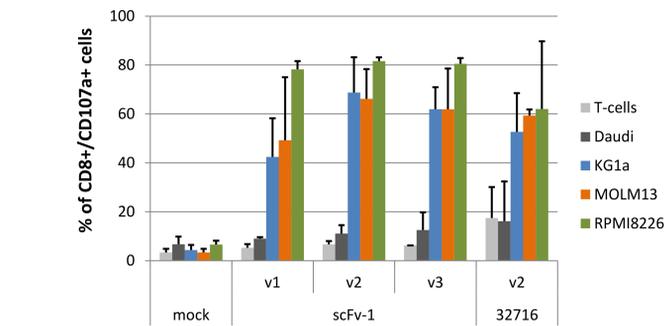
An scFv targeting the extracellular domain of the human CD123 antigen was used to construct three chimeric antigen receptors, each containing hinge sequences of different lengths.



#5 CD123 CAR T-cell in vitro activity

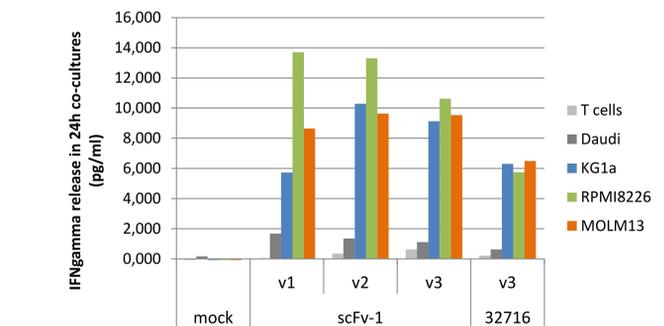
T-cells stably expressing the three CAR constructs and a benchmark CAR (32716) were generated by lentiviral transduction and their activity was assessed 10 days after transduction.

DEGRANULATION ASSAY



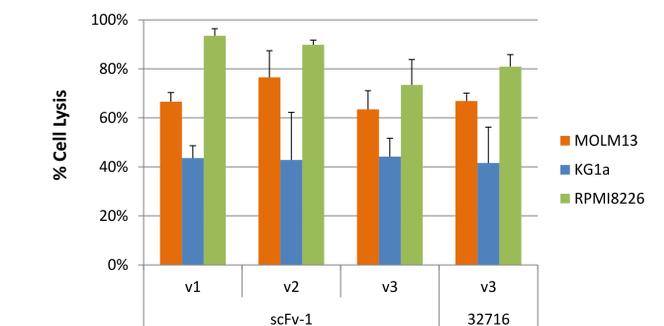
CAR+ T-cells were co-cultured with CD123+ target cells (1:1). Degranulation activity was measured by staining with CD8 and CD107a antibodies and analyzed by flow cytometry. Daudi cells were used as a negative control.

IFNgamma RELEASE ASSAY



CAR+ T-cells were co-cultured with CD123(+) target cells (1:1). The amount of IFN γ released in the culture supernatants was determined by ELISA.

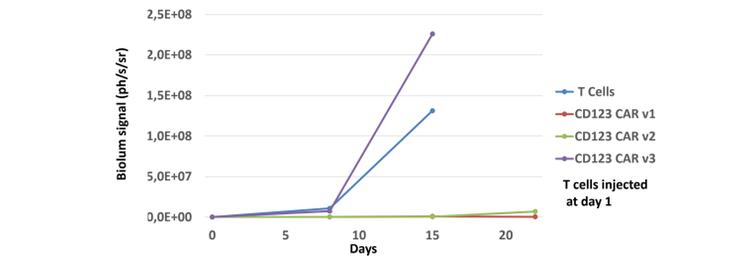
CYTOTOXICITY ASSAY



CAR+ T-cells were co-cultured with CD123(+) target cells and CD123(-) cells (10:1:1 ratio). The viability of each cell line -CD123(+) and CD123(-) was determined after 4h of co-culture to determine the % of specific cell lysis.

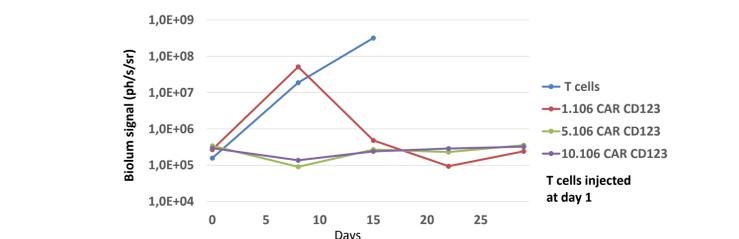
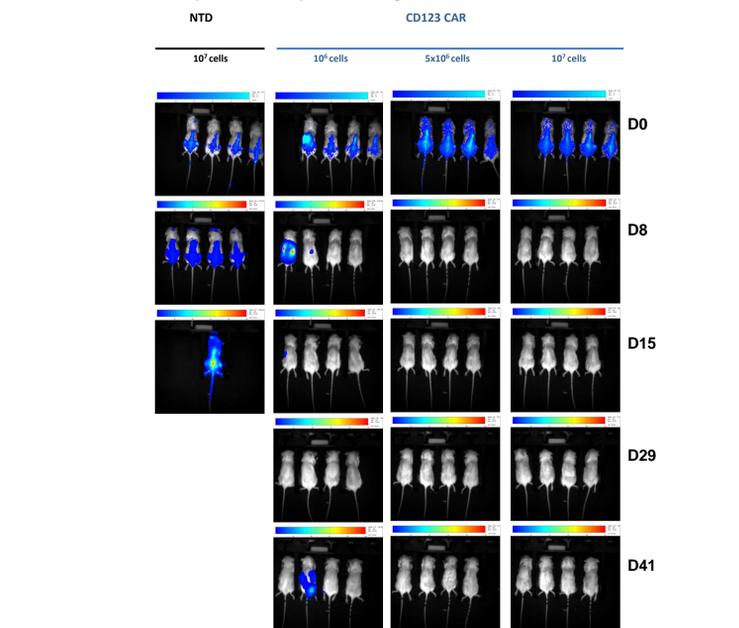
#6 CD123 CAR T cell in vivo anti-tumor activity

All three CAR constructs were evaluated for vivo anti-tumor activity: 5 x 10⁶ CAR-positive T-cells were intravenously injected in NSG mice 7 days after tumor cell injection (2.5 x 10⁵ Molm13-ffluc cells).



The CD123 CAR v1 and v2 display a strong anti-tumor effect while the CD123 CAR v3, despite significant in vitro activity, did not display any anti-tumor activity in vivo.

The anti-tumor activity of the v2 CAR was further characterized in a long term anti-tumor study: several doses of CAR-positive T-cells (1 x 10⁶, 5 x 10⁶ and 10 x 10⁶ cells) were intravenously injected in NSG mice 7 days after tumor cell injection (2.5 x 10⁵ Molm13-ffluc cells) and mice were followed for up to 60 days. The T cells were injected at Day 1 in the figures below.



Except for one mouse (treated at 1x10⁶), all the mice treated with CAR T-cells were alive at Day 60. In addition, flow cytometry analysis at Day 60 confirmed the complete elimination of the tumor cells in the blood, the spleen and the bone marrow.

UCART19, an Allogeneic “Off-the-Shelf” Adoptive T-cell Immunotherapy against CD19⁺ B-cell Leukemias



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#1 Abstract

Background: Autologous T-cells engineered to express chimeric antigen receptors (CARs) that target specific tumor antigens are of high potential in treating different kinds of cancer. However, they must be generated on a “per patient” basis, thereby limiting the population of patients that could benefit from this approach.

Methods: We have developed a standardized platform for manufacturing T-cells from third-party healthy donors to generate allogeneic “off-the-shelf” engineered CD19-CAR+ T-cell-based frozen products. Our platform involves the use of transcription activator-like effector nucleases (TALEN), which mediate the simultaneous inactivation of two genes through genome editing. The knockout of the TCR alpha gene eliminates TCR expression and is intended to abrogate the donor T-cell’s potential for graft-versus-host disease (GvHD), while knocking out the CD52 gene makes donor T-cells resistant to the lymphodepleting agent alemtuzumab. In addition, our T-cells are engineered to co-express the RQR8 gene as a safety feature, with the aim of rendering them sensitive to the monoclonal antibody rituximab.

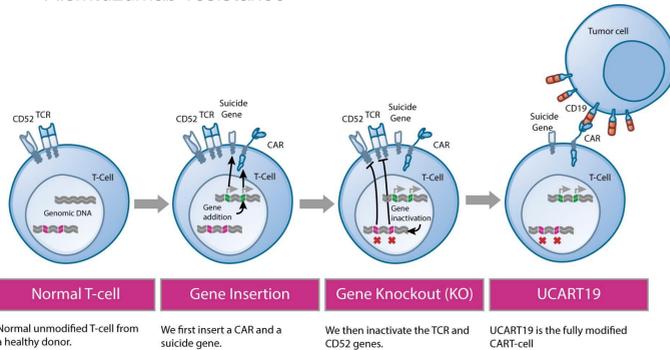
Results: We have obtained proof-of-concept by manufacturing TCR/CD52-deficient RQR8+ and CD19-CAR+ T-cells (UCART19) using a good manufacturing practice-compatible process and have demonstrated that UCART19 cells were functional using in vitro assays. Furthermore, we have demonstrated that the ability of UCART19 cells to engraft into an orthotopic human CD19+ lymphoma xenograft immunodeficient mouse model. UCART19 cells exhibited antitumor activity equivalent to that of standard CD19 CAR T-cells. We also demonstrated that UCART19 cells did not mediate alloreactivity in a xeno-GvHD mouse model. Finally, the effectiveness of the rituximab-induced depletion mechanism of RQR8+ cells was shown in an immunocompetent mouse model.

Conclusions: This valuable dataset supports the development of allogeneic CAR T-cells, and UCART19 will be investigated in an exploratory, first-in-human, clinical trial where refractory/relapsed CD19+ B-cell leukemia patients are to be enrolled.

#2 UCART19: an allogeneic CAR T cell for ALL and CLL

Cell Development Process:

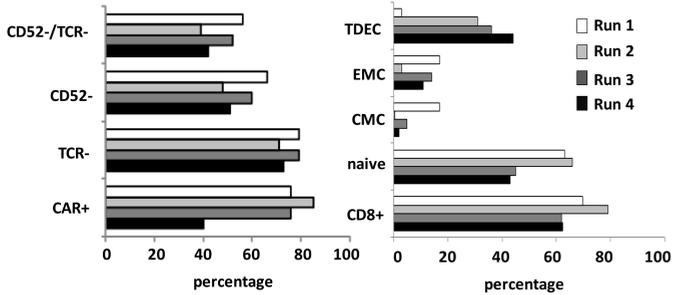
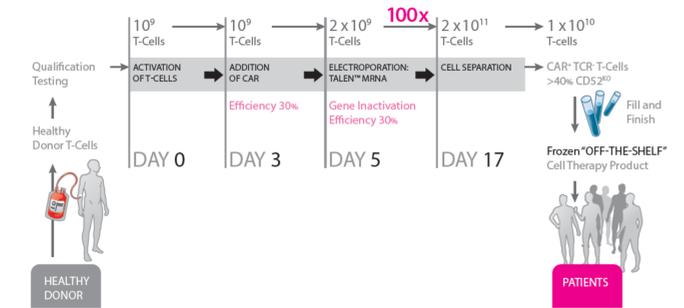
1. Start from healthy, unmodified donor T-Cells
2. Insertion of single-chain CAR construct and suicide gene
3. Gene-knockout of TCR alpha to avoid GvHD and CD52 for Alemtuzumab resistance



The key barriers to the adoptive transfer of 3rd party CAR T-cells can be overcome via application of TALEN gene editing technology - deletion of the TCR gene allows use of any donors’ T-cells without the potential for GvHD, while deletion of CD52 allows for resistance to the lymphodepleting agent alemtuzumab.

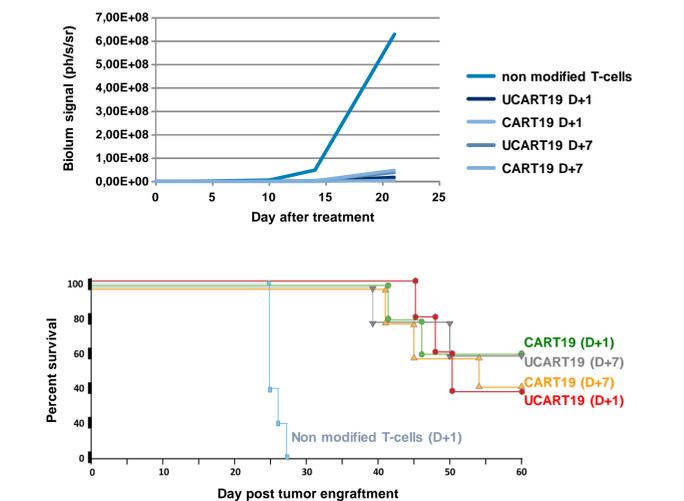
#3 Optimized UCART19 T cell Manufacturing Process

Collectis has developed and optimized a 17 day T-cell manufacturing process permitting production of TCR/CD52-deficient CAR T-cells with highly reproducible yields and cell phenotypes.



#4 UCART19 cells display potency equivalent to CAR T cells

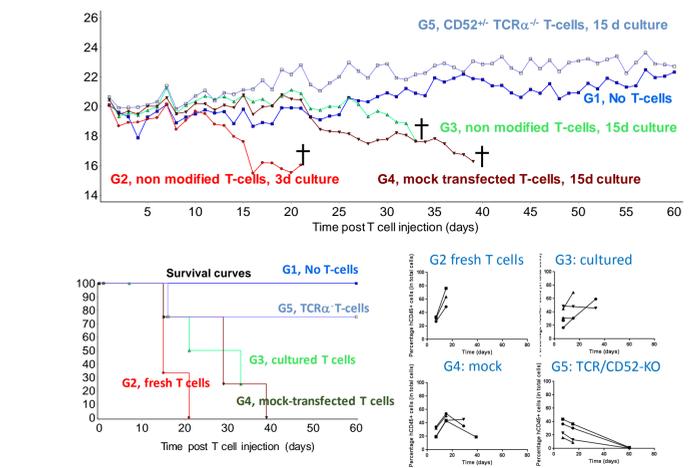
Anti-tumor activity: 4x10⁶ CAR-positive T-cells (UCART19 or regular CART19 cells) were intravenously injected in NSG mice either 1 day (5x10⁵ Daudi-ffluc cells) or 7 days (2.5x10⁵ Daudi-ffluc cells) after tumor cell injection. Mice were serially monitored for the first 3 weeks via luminescence imaging to follow tumor progression, and over an 8 week period for survival.



UCART19 cells display a potency that is equivalent to standard CAR T cells indicating that the inactivation of TCR alpha and CD52 genes has no major impact on anti-tumor activity.

#5 TCR alpha inactivation prevents Graft vs. Host Disease

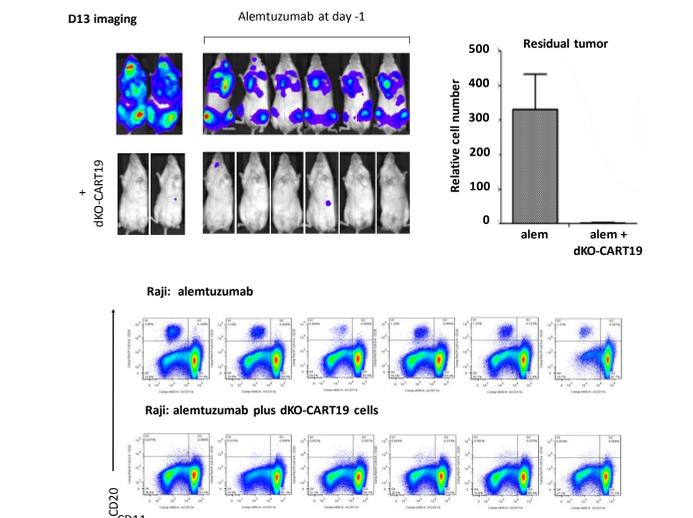
Irradiated NOG mice were injected with PBS (group 1), or 30x10⁶ human T cells from the same donor. The cells were obtained from activated PBMCs after 3 day expansion (group 2), 15-day expansion (group 3), mock electroporation and 15-day expansion (group 4), or CD52/TRAC TALEN-electroporation, 15-day expansion and depletion of TCR+ cells (group 5).



No mice receiving TRAC/CD52 TALEN-treated cells showed signs of GvHD whereas all mice receiving non engineered cells developed GvHD and had to be sacrificed. Human cells survived at least 2 weeks in all groups.

#6 UCART19 cells display efficient anti-tumor activity in presence of alemtuzumab

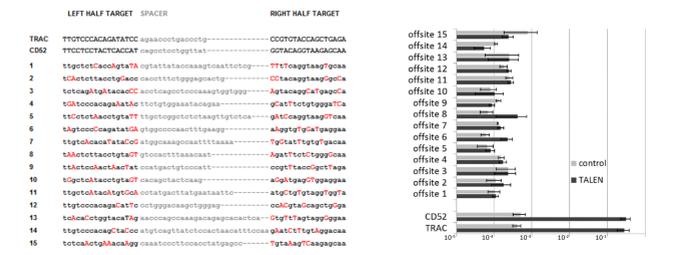
Anti-tumor activity in the presence of alemtuzumab: Alemtuzumab was injected into NSG mice one day prior to CD52^{+/+} Raji-ffluc cell injection (2.5x10⁵ CD52^{+/+} Raji-ffluc, 30% CD52-deficient), and dKO-CART19 cells were intravenously injected the following day.



NSG mice treated with Alemtuzumab combined with UCART19 cells display complete elimination of Raji-ffluc cells from their preferred engraftment niche, bone marrow, as assessed by luminometry or flow cytometry of cell suspensions obtained from bone marrow isolated at 14 days post-injection.

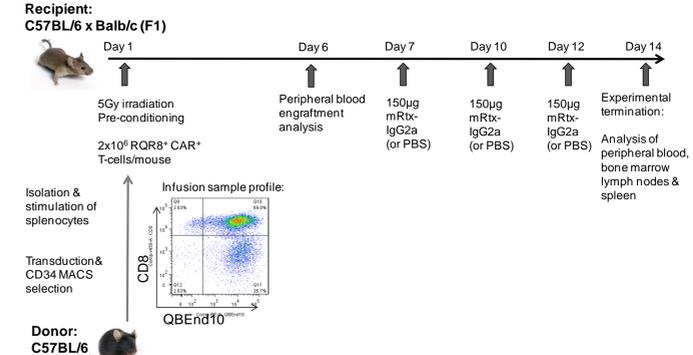
#7 Absence of off target cleavage in UCART19 cells

Using recent data quantifying the impact of a substitution in a target sequence on the efficiency of TALEN cleavage we identified the top 15 potential off-target sequences in the human genome. Using deep sequencing analysis, we then examined the frequency of mutations found at these loci in UCART19 cells.

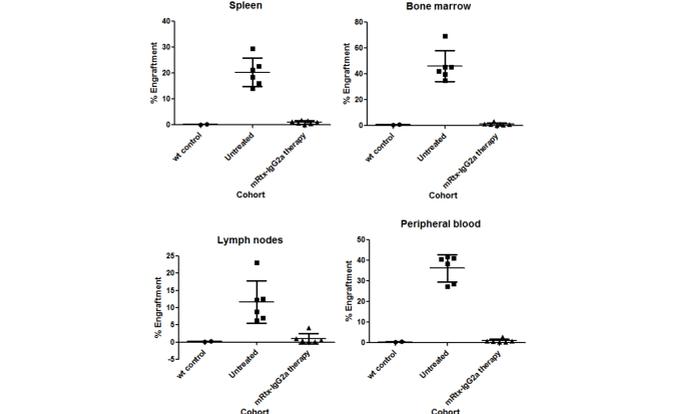


The highest observed frequency of insertion/deletion at any of the offsite targets was 1 in 8x10⁻⁴ (black bars)—a rate that is statistically indistinguishable from what we observe in control amplicons (gray bars). These results demonstrate an exceptionally low rate of offsite target cleavage—at least 600 times less likely than the intended targets.

#8 Efficient depletion of RQR8 positive cells in vivo



The susceptibility to Rituximab of RQR8-positive cells was shown *in vivo* in an immunocompetent mouse model using a re-engineered Rituximab to a mouse IgG2a, the functional equivalent of human IgG1 (mRtx-IgG2a) (Philip et al., 2014).



Flow cytometry analysis at Day 14 of spleen, bone marrow, lymph nodes and blood indicate a significant depletion of RQR8+ cells in mice treated with Rituximab.